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PRINCIPAL INVESTIGATOR: Michael Chancellor

CONTRACTING ORGANIZATION: Lipella Pharmaceuticals, Inc.
Pittsburgh, PA 15208

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14. ABSTRACT
Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating disorder characterized by persistent pelvic pain associated with bladder symptoms including urinary frequency and urgency. Previous studies have indicated that overexpression of nerve growth factor (NGF) is an important factor in the symptom development of IC/BPS. This project evaluates the potential of an anti-NGF bladder drug delivery system as a potential investigational drug product indicated for IC/BPS. The investigational drug product is a proprietary liposome NGF-antisense formulation given the development name of LP-11. The project aims included research, manufacture, and nonclinical testing of LP-11. The long-term objective of the research program is to establish new local anti-NGF therapy for the treatment of IC/BPS.

15. SUBJECT TERMS
Interstitial cystitis/painful bladder syndrome (IC/PBS), Liposome

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I-1. Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a debilitating disorder characterized by persistent pelvic pain with potential symptoms of urinary frequency, urgency, nocturia, and painful intercourse. IC/BPS can affect both genders with epidemiological data suggesting that up to 12% of women may have early symptoms of IC/BPS. IC/BPS patients are currently treated through various means: pentosan polysulfate sodium, dimethyl sulfoxide, off label use of amitriptyline, anesthetics, immunosuppressive drugs and narcotic analgesics. Each of these approaches has significant limitations. Unfortunately, there are currently no adequate medical or surgical remedies for many IC/BPS patients. IC/BPS is recognized as a significant unmet medical need.

Previous studies indicate overexpression of nerve growth factor (NGF) as a key factor in the symptom development of IC/BPS. NGF antisense oligonucleotides hold promise for IC/BPS by restricting the effect of NGF in the bladder. However, a systemic approach for NGF silencing in the bladder risks inducing systemic adverse events, suggesting a local-bladder drug delivery approach is needed. Local delivery options have traditionally been hampered by inefficient intracellular delivery of antisense. Liposomes have been used for drug delivery in a variety of applications. The investigators have previously investigated liposome drug delivery formulations and have completed studies using intravesical liposomes to demonstrate they are not systemically absorbed.

This project explores the feasibility of liposome mediated delivery of anti-nerve growth factor (NGF) for bladder pain and frequency. Investigators are developing an investigative product, called LP-11, consisting of a liposome vehicle mixed with antisense for intravesical delivery, with LP-11's formulation under development in this project. The goal of this project is to provide the foundation for future translation of LP-11 and local NGF antisense therapies for the IC/BPS indication. Lipella Pharmaceuticals Inc collaborated with investigators located at the University Of Pittsburgh School Of Medicine to conduct the experimental program.

I-2. Keywords

Interstitial cystitis/bladder pain syndrome (IC/BPS), nerve growth factor (NGF), small interfering RNA (siRNA), liposome, urology, drug delivery

I-3. Overall Project Summary

In this project, investigators worked to develop a local therapy targeting NGF production in the bladder for the treatment of bladder pain. Aims of this project support the preclinical investigation and early product development of LP-11. The broad aims of the project are to characterize, develop manufacturing methods, and conduct formulation development of LP-11 for future clinical trials. The project aims also include preclinical evaluation of LP-11 in experimental rat animal models. The approved statement of work (SOW) for the experimental plan and timeline are is listed in the table below (Section II-1).

II. Accomplishmnets
II-1. Timeline described in the SOW

	Year 1		Year 2			Year 3		
	0-4mo	5-12mo	13-16mo	17-20mo	21-24mo	25-28mo	29-32mo	33-36mo

AIM 1	□ Regulatory approval for animal research
	□ Obtain written approval from both IACUC and ACURO
		□ Order animals/
		□ Prepare protocols/ documentation
		□ Initiate animal study and confirmation of CYP and TNBS
			□ CYP, TNBS treatment
			□ LP-NGF antisense treatment
			  o Pain behavior/bladder over-activity assessment
			□ Tissue Analysis
			  o PCR/ELISA
			  o Patch Clamp
						Complete animal studies	Animal study data analysis/reporting	

AIM 2	□ Optimize formulation □ Manufacture LPs □ Analytical method development		□ Develop GMP LP-NGF antisense manufacture process □ cGMP documentation reporting □ Manufacture GMP LP □ Product specifications/batch release testing □ Biosdistribution assessment			□ Stability Testing □ Ex-vivo stress testing		

II-2. Research Accomplishment Description
AIM 1 *Regulatory approval for animal research; Obtain written approval from both IACUC and ACURO*
[Accomplishment]
Initial IACUC approval was received in January 2012 by the University of Pittsburgh Institutional Animal Care and Use Committee. Furthermore, ACURO approval of the same protocol was confirmed on Thursday, January 10, 2013
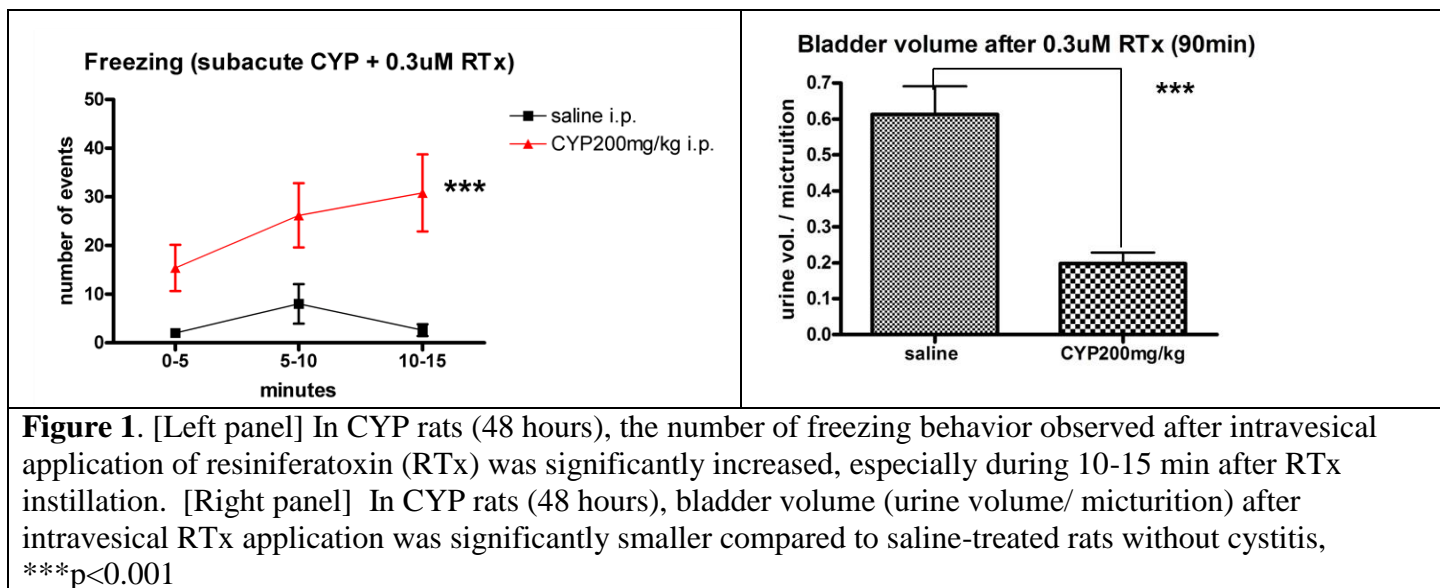
AIM 1 *Confirmation of CYP*
[Accomplishment]
1. Confirmation of CYP cystitis model
Co-investigators at the University of Pittsburgh examined whether chemical cystitis induced by cyclophosphamide (CYP) enhance nociceptive responses to resiniferatoxin (RTx) stimulation to confirm that the CYP model is suitable for bladder pain research. Saline or CYP200mg/kg was injected intraperitoneally to female SD rats. Two days later, in an awake condition, 0.3μM RTx (0.3ml, 1 min) was injected into the bladder through a urethral catheter to evaluate nociceptive behaviors such as freezing (motionless head-turning) that were counted and recorded every 5 seconds for 15 minutes. Urine volume and frequency were recorded simultaneously in metabolic cages.

We have found that: (1) freezing behavior, which reflects bladder pain, induced by RTx was significantly increased in CYP cystitis vs. control rats and (2) bladder volume (urine volume/ micturition) was significantly decreased in CYP cystitis vs. control rats (Figure 1). These results indicated that CYP rats have bladder pain associated with frequent voiding, which are the main symptoms of IC/BPS.
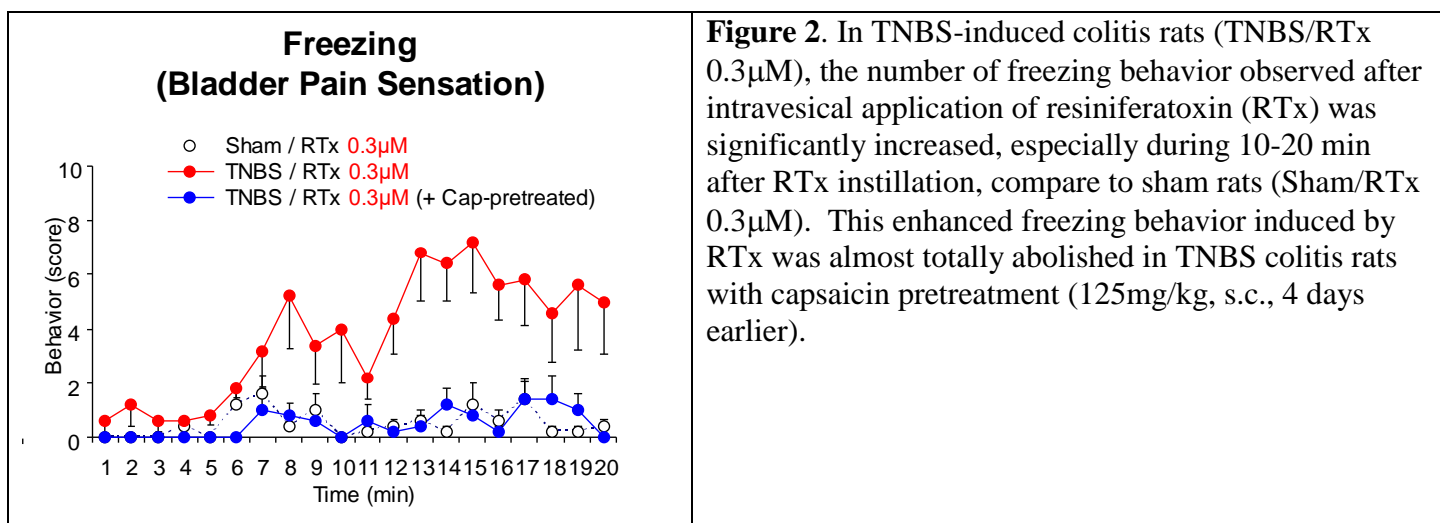
Figure 1. [Left panel] In CYP rats (48 hours), the number of freezing behavior observed after intravesical application of resiniferatoxin (RTx) was significantly increased, especially during 10-15 min after RTx instillation. [Right panel] In CYP rats (48 hours), bladder volume (urine volume/ micturition) after intravesical RTx application was significantly smaller compared to saline-treated rats without cystitis, ***p<0.001

AIM 1 *Confirmation of TNBS colitis model*
[Accomplishment]
Co-investigators also examined whether pain behavior in response to bladder irritation was enhanced in rats with experimental chronic colitis to confirm that the model would be suitable for bladder pain research. Experimental colitis was induced by intracolonic injection of trinitrobenzene sulfonic acid (TNBS 50 mg/mL in 50% ethanol, 0.4mL) through a transanally placed. Ten days later, in an awake condition, 0.3μM RTx (0.3ml, 1 min) was injected into the bladder through a urethral catheter to evaluate nociceptive behaviors such as freezing that were counted and recorded every 5 seconds for 20 minutes (n=5 each).

We have found that; (1) freezing behavior, which reflects bladder pain, was significantly increased in TNBS colitis vs. control rats, (2) increased freezing behavior in colitis rats was suppressed when C-fiber afferent pathways were desensitized by capsaicin pretreatment (Figure 2) and (3) the NGF protein level in the mucosa containing urothelial cells was significantly (p<0.05) increased in colitis rats (261.8 pg/mg total protein, n=4) compared to sham rats (136.8 pg/mg, n=5). These results indicated that TNBS colitis rats have bladder pain due to activation of C-fiber afferent pathways, which is associated with NGF upregulation in the mucosa.

Figure 2. In TNBS-induced colitis rats (TNBS/RTx 0.3μM), the number of freezing behavior observed after intravesical application of resiniferatoxin (RTx) was significantly increased, especially during 10-20 min after RTx instillation, compare to sham rats (Sham/RTx 0.3μM). This enhanced freezing behavior induced by RTx was almost totally abolished in TNBS colitis rats with capsaicin pretreatment (125mg/kg, s.c., 4 days earlier).

AIM 1 *CYP, TNBS treatment: Validation of the colitis models and development of the new animal model of chronic cystitis induced by hydrogen peroxide*
[Accomplishment]

4

We have found that; (1) TNBS colitis rats exhibit enhanced freezing behavior that reflects bladder pain, which was suppressed by desensitization of C-fiber afferent pathways by capsaicin pretreatment, (2) 7-14% of L1, L6 and S1 DRG neurons innervate both colon and bladder, (3) the TRPV1 mRNA level is increased in S1 DRG and (4) an increase in MPO activity that reflect the level of neutrophil infiltration was seen in the colon, but not in the bladder. These results indicated that TNBS-induced colitis increased pain sensitivity in the bladder via activation of C-fiber afferent pathways due to colon-to-bladder cross-sensitization (Yoshikawa et al., 2014).

Furthermore, we examined changes in colon and bladder activity after the application of allyl isothiocyanate (AI), a TRPA1 channel activator, into the colon in rats (Furuta et al., 2014). We found that: (1) the intracolonic AI application, which significantly increased mean intracolonic pressure, induces bladder overactivity due to colon-to-bladder cross-sensitization, which is prevented by pretreatment with HC-030031, a TRPA1 inhibitor and (2) Evans blue dye extravasation is significantly increased in the AI-treated inflamed colon and also in the bladder following intracolonic AI treatment. These results indicate that TRPA1 channels expressed in C-fiber afferent pathways greatly contribute to colon-to-bladder cross-sensitization to induce bladder nociceptive responses such as bladder overactivity (Furuta et al., 2014).

Additionally, previous work in phase 1 lead itself to research conducted by the investigators concurrently during this period, which looked at the therapeutic effect of a sensory neuron-specific receptor (SNSR) agonist (BAM8-22) on bladder overactivity in a rat model of cystitis induced by cyclophosphamide (CYP), which was verified as a cystitis model with urothelial molecular changes in this project (Sugino et al., 2015), and found that activation of SNSR can suppress CYP-induced bladder overactivity, due to suppression of bladder afferent activity, suggesting that the SNSR is a potential target for the treatment of bladder hypersensitive disorders such as IC/PBS (Honda et al., 2014). Also, using patch-clamp recording techniques, we reported that different types of voltage-gated potassium channel -subunits such as Kv1.4 or Kv4.1/4.3 subunits are involved in the control of neuronal excitability in somatic and visceral afferent neurons including bladder afferent cells in L6-S1 DRG of rats (Yunoki et al., 2014).

Furthermore, in addition to a CYP-induced cystitis model, we sought to establish a long-lasting cystitis model induced by intravesical instillation of hydrogen peroxide (H2O2). Using female rats, 1.5% H_2O_2 solution was introduced transurethrally into the bladder of female rats, and kept for 30 min. We have found that; (1) the H_2O_2 injection increased the number of micturition events up to day 14 and decreased urine volume per micturition, (2) intercontraction intervals were shortened without affecting the baseline, threshold, or maximum pressures, (3) RTx-evoked nociceptive behaviors, such as freezing, were enhanced on days 7 and 14 and (4) histopathology revealed hemorrhage, edema, infiltration of neutrophils into the lamina propria, and urothelial denudation in the early phase, and these damages were gradually repaired, while hyperplasia of the urothelium, vascularization, increases in fibroblast counts, and infiltration of mast cells and eosinophils were observed through the later phase. These results indicated that intravesical H_2O_2 injection induces relatively long-lasting cystitis with enhanced bladder activity and pain sensation in rats. These results are reported in a recently accepted manuscript for publication in Physiological Reports (Dogishi et al., 2017)

AIM 1 *Evaluate LP-NGF antisense treatment-confirmation of methods*
[Accomplishment]
We first performed in-vivo experiments to test the efficacy of LPs conjugated with NGF antisense using an acute cystitis rat model. Under isoflurane anesthesia, rats were catheterized by a 24-gauge angiocatheter through the urethra into the bladder.   After urine was drained from the bladder, 12μM of NGF antisense or scramble ODN complexed with liposome or saline in a volume of 0.5ml was infused for 30 minutes.   The efficacy of LP-antisense treatments was assessed 24h after infusion by saline and subsequent acetic acid (AA) cystometry under urethane anesthesia. A control cystometrogram (CMG) was performed during filling the bladder with saline to elicit repetitive voiding more than for 1 hour followed by 0.25% AA infusion to induce bladder irritation for more than 3 hours. The intercontractile interval (ICI) of the reflex bladder contractions during saline and AA was measured and compared.

We have found that; (1) LPs complexed with NGF antisense were retained in the urothelium after intravesical application as evidenced by histological identification of LP antisense tagged with a fluorescent dye (Kashyap et al, 2013), (2) LPs-NGF antisense treatment suppressed AA-induced bladder overactivity as evidenced by the reduction in the ICI decrease after intravesical AA application in the LPs-NGF-treated groups vs. control groups (saline or LPs-scramble oligo treatment), and (3) LPs-NGF antisense treatment reduced the NGF expression in the bladder mucosal layer (Kashyap et al, 2013). We also reported that NGF antisense intravesical treatment can reduce the expression of NGF and an NGF receptor p75 in the rat bladder (Kashyap et al., 2016). Taken together, these results indicate that the LPs-NGF antisense complex is effective to suppress the urothelial NGF expression and inhibit bladder overactivity induced by bladder afferent sensitization (Yoshimura et al., 2014; Tyagi et al., 2014).

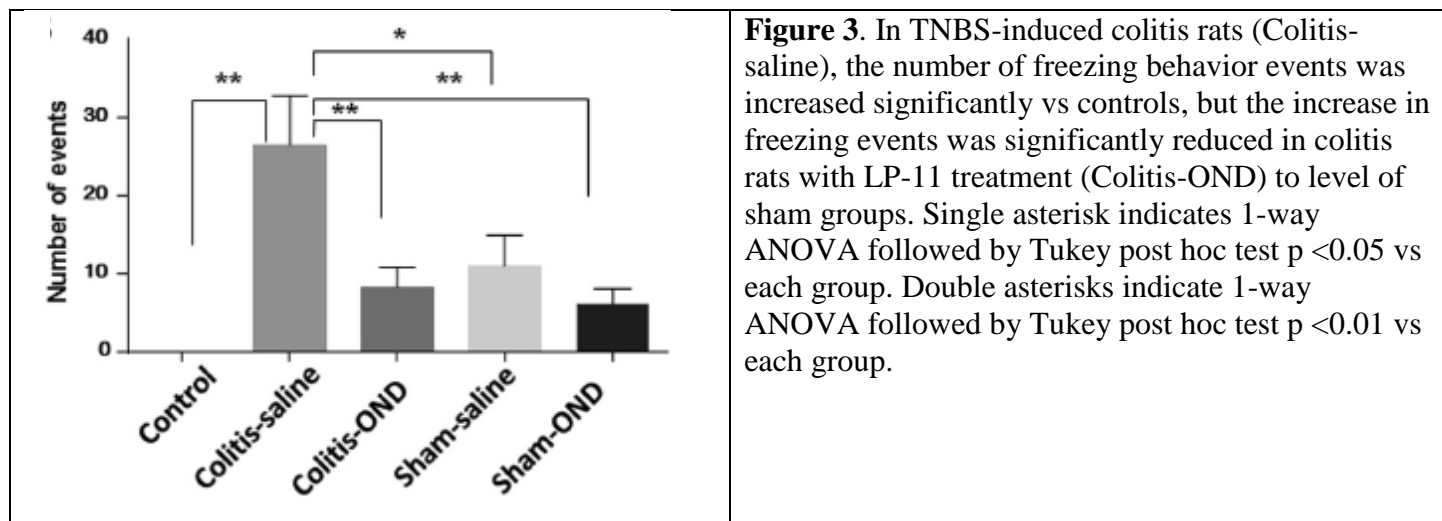**AIM 1** *Evaluate LP-NGF antisense treatment-colitis model*
DBased on the findings of the acute cystitis model, we completed the experiments that examined the therapeutic effects of intravesical liposome-NGF antisense treatment experiment in the TNBS colitis model.

Purpose: The purpose of the study was to evaluate the effect of bladder delivery (intravesical) of liposome conjugated with LP-11 in a rat model of IC/BPS symptoms and NGF expression induced by intracolonic 2,4,6,trinitrobenzen sulfonic acid enema (TNBS) experimental colitis.

Methods: Adult female Sprague-Dawley rats were divided into five groups; (a) control group (no TNBS and no LP-11), (b) a TNBS and LP-11 treated group, (c) a TNB treated with intravesical placebo (saline) group, (d) sham (no TNBS) and LP-11 treated group and (e) sham-saline group. Intravesical administration of .2ml of LP-11 or saline was given under isoflurane anesthesia was instilled to the bladder through an inserted urethral catheter prior to TNBS administration. Twenty-four hours after instillation of LP-11 or saline, colitis was induced by the enema of 30mg TNBS.

Ten days after LP-11 or saline injection treatments, animals were subjected to either in vivo studies or bladder tissue removal. Testing involved nociceptive behavior testing for pain, cytometry for frequency (ICI), immunohistochemistry, and polymerase chain reaction for NGF analysis.

Summary of Results: (1) in the TNBS-saline group, the score of freezing behavior, defined as a lack of movement by the rats representing bladder pain sensation, was significantly higher than that of other groups including the TNBS-LP-11 group (Figure 3); (2) the ICI reduction rate into the bladder is significantly higher in the TNBS-saline group than that in the TNBS-LP-11 group; (3) The mRNA and protein expression of NGF in the mucosa were significantly higher in the TNBS-saline group compared to the TNBS-LP-11 group. These finding suggest a beneficial effect of LP-11 on IC/BPS according to the measures.

Figure 3. In TNBS-induced colitis rats (Colitis-saline), the number of freezing behavior events was increased significantly vs controls, but the increase in freezing events was significantly reduced in colitis rats with LP-11 treatment (Colitis-OND) to level of sham groups. Single asterisk indicates 1-way ANOVA followed by Tukey post hoc test p <0.05 vs each group. Double asterisks indicate 1-way ANOVA followed by Tukey post hoc test p <0.01 vs each group.

Protein Expression was evaluated in the bladder which was dissected to divide into mucosal and detrusor layers for quantification of protein of NGF. Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to measure the protein expression of NGF. We observed the protein expression of NGF in the TNBS-saline group was significantly increased in the mucosa compared to control and TNBS-LP-11 groups. LP-11 reduced protein levels due to the direct antisense effect of reduced transport of NGF from the bladder to bladder afferent pathways in TNBS rats

Conclusions: Our overall conclusions that LP-11 had a therapeutic effect on TNBS-induced bladder hypersensitivity as evidenced reductions in licking behavior and NGF protein expression compared to placebo are unchanged given these results. Preclinical efficacy studies suggest LP-11 may be a promising treatment for IC/BPS. The results were presented at the Military Health Research Symposium (MHSRS) in August, 2015 (Chancellor et al, 2015, MHSRS abstract) as well as in our recent review articles (Ogawa et al., 2015; Tyagi et al., 2016) and have been published in in Journal of Urology (Kawamorita et al., 2016).

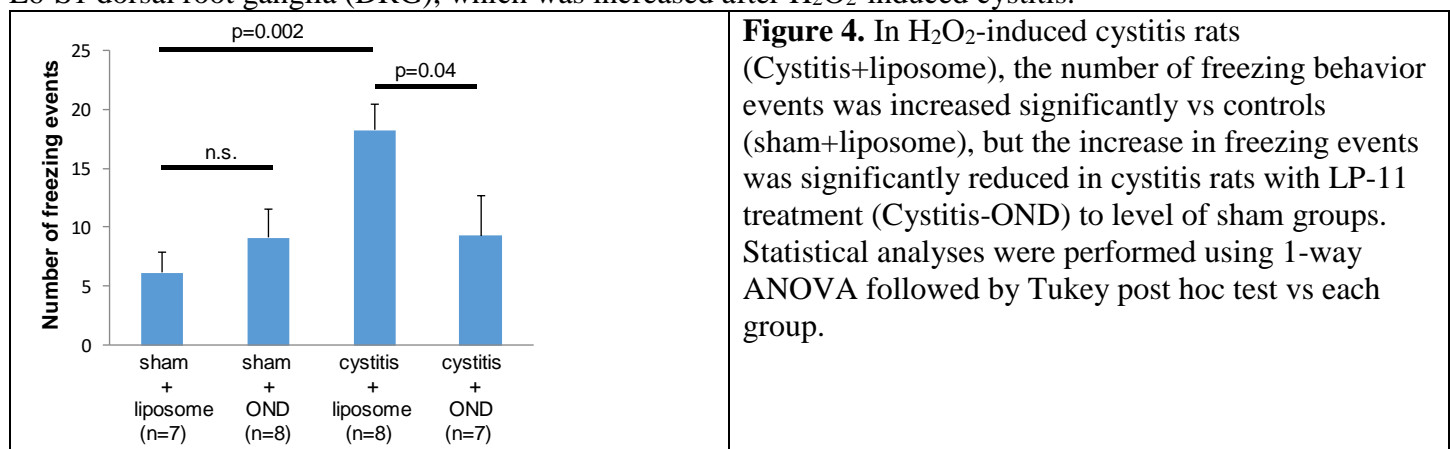**AIM 1** *Evaluate LP-NGF antisense treatment-cystitis model*
We also examined the therapeutic effects of intravesical liposome-NGF antisense treatment experiment in the H_2O_2 cystitis model.
Although we showed that CYP-induced cystitis rats exhibited bladder overactivity and increased pain sensitivity, the expression of NGF in the bladder was not always stable. Therefore, we improved the model of chronic cystitis induced by intravesical application of H_2O_2 (Dogishi et al., 2017), and tested the efficacy of LP-11 treatment targeting bladder NGF upregulation in this chronic cystitis model.

Purpose: The purpose of the study was to investigate the effect of intravesical liposome-based NGF antisense (LP-11) on bladder overactivity and nociceptive behavior in a rat model of chronic cystitis induced by intravesical H_2O_2 instillation.

Methods: Adult female Sprague-Dawley rats were divided into five groups; (a) saline + vehicle (SV) group, (b) saline + LP-11 group, (c) 1.5% H_2O_2 + vehicle group, (d) 1.5% H2O2 + LP-11 group. Saline or 1.5% H_2O_2 was administered into the bladder on day 0. Each rat was treated with intravesical vehicle or NGF antisense administration on day 2.

Summary of Results: (1) H_2O_2-induced cystitis rats with LP-11 treatment showed significantly less bladder overactivity (cytometry) and reduced bladder-related pain behavior (freezing behavior) compared with cystitis rats without LP-11 treatment (Figure 4). (2) HE staining of bladder sections showed that substantial infiltration of the inflammatory cells, submucosal bleeding, and detrusor hypertrophy seen in H2O2-induced cystitis rats; however these histological changes were alleviated by the LP-11 treatment. (3) In RT-PCR, H_2O_2-induced cystitis rats with LP-11 treatment showed the reductions in expression of NGF and TRPV1 in the mucosa and L6-S1 dorsal root ganglia (DRG), which was increased after H_2O_2-induced cystitis.

Figure 4. In H_2O_2-induced cystitis rats (Cystitis+liposome), the number of freezing behavior events was increased significantly vs controls (sham+liposome), but the increase in freezing events was significantly reduced in cystitis rats with LP-11 treatment (Cystitis-OND) to level of sham groups. Statistical analyses were performed using 1-way ANOVA followed by Tukey post hoc test vs each group.

Conclusions: These results indicate that intravesical LP-11 therapy induces a reduction in the NGF expression in the bladder mucosa and bladder afferent pathways, which results in the improvement of bladder pain behavior and frequent urination induced by hydrogen peroxide-induced chronic cystitis. Thus, intravesical LP-11 therapy could be a novel treatment that can avoid systemic adverse events for hypersensitive bladder disorders such as IC/BPS, in which NGF has been implicated as an important mediator for inducing afferent sensitization. These results were presented at 2015 International Continence Society (ICS) meeting in Montreal, Canada (Majima et al., 2015; ICS abstract) and 2016 AUA meeting in San Diego (Majima et al., 2016; AUA abstract), and have been reported in a recently accepted paper for publication in Human Gene Therapy (Majima et al., 2017).

AIM 2 *Manufacture LP-11*
[Accomplishment]
We formulated and manufactured liposomes (LPs) conjugated with NGF antisense. The 18mer phosphorothioate oligodeoxynucleotide (ODN) with the sequence 5'-GCCCGAGACGCCTCCCGA-3' for the experiments were made, and cationic liposomes composed of DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N trimethylammonium methylsulfate) were made by thin film hydration method and hydrated with nuclease free water with the final lipid concentration of 7mM. The ODN were dissolved in nuclease free water at the concentration of 2mM and were complexed with liposomes in the proportion of 6μl ODN solution to 1 ml liposome lipid by incubation at room temperature.

Lipella has developed a scalable manufacturing method for LP-11. Specific procedures for handling, processing, environmental control, identification, packaging, testing, storage, and release of the company's product were created. Standard operating procedures (SOPs) have been put in place for the full manufacturing method. At this time, liposomal processing at Lipella includes aseptic handling under procedurally controlled conditions to prevent contamination and to preserve function and integrity of an LP-11 experimental drug.

LP-11 material sourcing with material companies for production and testing was initiated with purchasing of oligonucleotides from Integrated DNA Technologies (IDT). IDT's manufacturing headquarters achieved ISO 9001:2008: Quality Management Systems certification in 2005 and ISO 13485:2003: Medical Device Quality Management Systems certification in 2008. Mass spectrometry is used for Quality Control verification of each oligonucleotide synthesized. Lipella purchases the lipids from Avanti Polar Lipids. The sterile water for injection USP is similarly purchased from Baxter Healthcare Corporation.

Final product is tested in accordance with Lipella SOPs for compliance with final product release specifications. To enhance aseptic capabilities, the Lipella manufacturing suite and adjacent gowning room were upgraded and cleaned with 6% Sterile Hydrogen Peroxide (6% Steri Perox, supplied by Veltek Associates Inc.) with a minimum contact time of 1 hour. All surfaces of cleanroom including walls, floor, furniture and equipment (table, stools), and materials are sprayed except those for which spraying is unsuitable. The inside of the lyophilizer as well as the ceiling containing HEPA filters are wiped clean with wipes saturated with 6% Hydrogen Peroxide.

During the this grant, three environmental monitoring and cleaning evaluations were performed using Contact Sterile TSA Plates for surface viable counts; a Met One Particle Counter model no. 804 for non-viable air sampling counts; and Sterile TSA settling plates for viable air counts. Non-viable air counts were measured for 60 seconds and settle plates were exposed for a minimum of 4 hours. Contact and settling plates were incubated at 35°C for 7 days. Selection of environmental monitoring locations was based on a risk assessment. Viable air sampling was performed in the areas immediately adjacent to critical zones where work is being done. Dimensions of the manufacturing room are 5x7x7' with an identical size gowning room adjacent and separated by soft-wall curtain. Due to the small size of the manufacturing and gowning areas, a grid profiling approach to contact plate sampling location and sample number is not appropriate. One contact plate was used for each of the walls, ceiling, floor, furniture, two used on table, and three on product processing equipment (one inside, top

and front). Similarly, one contact plate was used for each of the walls, ceiling, floor and table in the gowning room. Contact plates were used to evaluate the sterility of operators' gloved fingertips following production. These assessments were reflected in evaluations results from the studies. No growth was observed on using plates during the three evaluation runs. Additionally, non-viable air sampling returned counts within limits for the classification of the room. These steps help ensure an acceptable environment for manufacturing LP-11. Furthermore, a class II SterilGAR hood was acquired with the goal to support future manufacturing of LP-11. We anticipate this addition will provide production scale up through anticipated early stage clinical trials of LP-11's development.

AIM 2 *Analytical method development*
[Accomplishment]
Lipella evaluated shelf life of LP-11 components using laser light scattering to determine particle size distribution of liposomes following freezer storage at -20°C as compared to the particle size distribution post-manufacture. Results from the particle size analysis noted that the test article can remain within release specifications following storage at -20°C. Physical stability of product was also assessed by noting lack of aggregation/precipitation following reconstitution. Additional evaluation of LP-11 shelf life testing with a sample stored at 40°C with relative humidity of 50-75% was performed.

Stress stability of liposomes was analyzed under two conditions: reconstitution with sterile water at room temperature and physiological stress conditions (reconstituted liposomes diluted in synthetic urine at 37°C). Separate batches were analyzed using microscopy, at room temperature for four hours to evaluate stability of reconstituted liposomes for clinical use and reconstituted liposomes in urine at 37°C for one hour to evaluate stress stability conditions of the urinary bladder. Microscopy video was taken for two separate batches of liposomes each in water at room temperature (4 hours) and in urine at 37°C (1 hour). Screenshots were taken at the beginning and end of each video and liposomes in both viewing windows were qualitatively compared with regard to size. In general, no observable changes in liposome size were recorded, indicating that the product relatively stable both in room temperature water and 37°C urine throughout the period in which they were tested.

AIM 2 *Product specifications/ Biodistribution assessment*
Development has been performed during the grant period to work on a method for determining the extent of encapsulation or entrapment volume of the siRNA antisense and the liposomes. Investigators in collaborating with a contract research organization and studied the total oligonucleotide content in phospholipid-based Lipo-siRNA formulation using High Performance Liquid—Strong Ion Exchange chromatography (HPLC-SAX) with UV detection.

Analysis of Formulation on As-Is Basis was performed first to determine signal of total oligonucleotide in the formulation. LP-11 powder was reconstituted in water for injection and was used to attempt the determination of the Oligonucleotide. Samples were analyzed on as-is basis and after addition of authentic Oligonucleotide. Triton X-100 was used to destroy (disrupt, dissolve) the micellar structure yielding a clear homogeneous solution. Additionally, samples of Lipo-siRNA reconstituted formulation were analyzed on as-is basis and after addition of Oligonucleotide authentic substance for verification of recovery and selectivity of the analytical methodology.

AIM 2 *Stability Testing*
 [Accomplishment]
LP-11 accelerated stability study:
A vial of DOTAP liposomal lyophilate was removed from the freezer and allowed to warm to ambient temperature. A 2 mM stock solution of SIRNA was made from dry, room temperature SIRNA powder with UltraPure Distilled Water (Invitrogen; RNAase and DNAase free). The DOTAP liposomal lyophilate was rehydrated, and an aliquot of the 2 mM SIRNA solution was added to the liposome suspension to make a 7 mg/mL DOTAP, 12 μM SIRNA solution. The resulting mixture was shaken for approximately 30 seconds.

250 µL of the resulting solution was added to 500 µL UltraPure water in a 2 mL tube, and another portion was added to 500 µL synthetic urine (Ricca Chemical Company) to bring the liposome concentration to 2 mg/mL. A sample was taken from each for baseline imaging. Each sample was placed in a water bath in an incubator at 37°C. Timing was started when the tubes were added to the incubator. Samples were taken for imaging at 30, 60, 90, 120, 180, and 240 minutes. Samples were diluted in their respective solvents to a 0.2 mg/mL liposome concentration for imaging. Images were sampled from three 3 µL spots on the microscope slide.
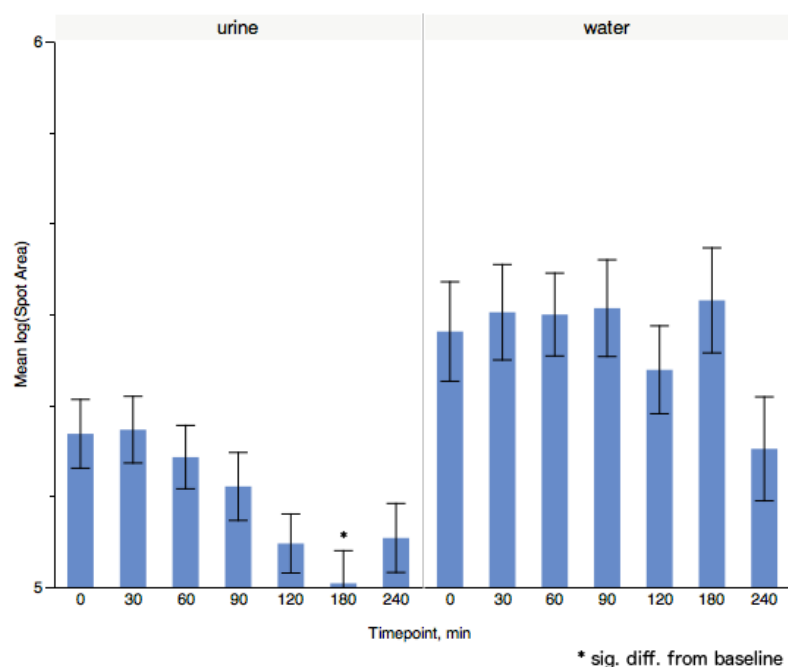
Figure 5. Accelerated Stability Study: Mean of log of particle areas. The urine and water groups differed significantly at each timepoint except for 240 min. The only significant change from baseline was in the urine group at 180 min.

For the accelerated stability study, a two-way ANOVA was performed with timepoint and diluent as factors (and the interaction of these factors). The effect of diluent (urine or water) alone suggests a significant difference in the distribution of spot sizes (p < 0.0001, mean log (urine) = 5.082, mean log (water) = 5.228, all timepoints). The effect of timepoint also shows a significant effect on the size distribution (p = 0.0194), and the factors do not significantly affect each other (p = 0.1984). Post-hoc analysis using Tukey's HSD test showed that, within each diluent group, only the 180 min point in the urine group was significantly lowered from baseline (p = 0.0278). The water distribution did not significantly change over time. T-tests between each diluent group at each timepoint were all significantly different (p < 0.05) except for at 240 min (p = 0.1457)

The following are summary statistics from the accelerated study:

	Synthetic Urine	Water
Timepoint	**Mean log(Spot Area) +/- SEM**	**Mean log(Spot Area) +/- SEM**
Baseline	5.14 +/- 0.03	5.23 +/- 0.05
30 min	5.14 +/- 0.03	5.25 +/- 0.04
60 min	5.12 +/- 0.03	5.25 +/- 0.04
90 min	5.09 +/- 0.03	5.26 +/- 0.04
120 min	5.04 +/- 0.03	5.20 +/- 0.04
180 min	*5.00 +/- 0.03	5.26 +/- 0.05
240 min	5.04 +/- 0.03	5.13 +/- 0.05

An interesting result to note here is that even though the same number of images was sampled for each solvent, there were fewer spots present in the water images. This is the reason for the slightly higher SEM. It is not clear if this is a physical effect or if it is due to under sampling. Because the SEM is consistently higher, it is likely not due to sampling. Because the liposomes in water are larger on average, it is possible that more of the

lipid material that would have made (more) smaller particles as in the case of the urine was instead incorporated in the (fewer) larger particles.

AIM 2: Long-term Stability of DOTAP liposomes LP-11 admixture
[Accomplishment]
Stability of the liposomal component of the LP-11 formulation was evaluated by testing two characteristics: DOTAP concentration and particle size.

A vial of DOTAP liposomal lyophilate was removed from the freezer and allowed to warm to ambient temperature. Sterile water for injection (WFI) was added to the vial to make a 2 mg/mL suspension of DOTAP. A sample from the vial was diluted to 0.2 mg/mL and three 3 µL sample spots were made on a microscope slide for imaging. This was performed at months 1, 2, 4, 6, and 12 after manufacture. A new vial was used at each timepoint.

Image Acquisition
All samples taken for imaging were diluted to 0.2 mg/mL liposome concentration. All microscope images were taken with an AmScope MA1000 camera at 1280x960 resolution with 120x magnification. At least 20 images were taken at each timepoint.

Image Analysis
The purpose of the analysis is to detect changes in the liposome size distribution over time (both studies) and between solvent groups (accelerated study). The metric that was measured is the number of pixels per liposome spot (i.e. particle area/size). Diameters and, by extension, liposome volumes *could* be estimated by taking a ratio of average spot diameter to a known reference spot, but this would introduce additional error since the liposome spots are not perfectly round in most cases and, as such, was not performed.

A program was written in the Python programming language using the scikit-image package to process the microscopy images and automatically obtain a distribution of particle areas for each image. The methodology is as follows:

Each microscopy image was obtained as a grayscale image. Each grayscale image is read into the program. The image pixel data are stored in a matrix with dimensions equal to the input image height and width. A Sobel filter is used to detect spot edges. The results of applying the Sobel filter are normalized to the range 0-255 (i.e. stored as an 8-bit grayscale image). A mean rank filter is applied to the Sobel-filtered image with a disk-shaped, 7-pixel radius, disk-shaped structuring element. This helps to reduce/eliminate bias introduced by illumination irregularities and to detect spot areas whose edges are defined by the Sobel filter. This structuring element was chosen after examining result from 3, 5, 7, 9, 12, and 15-pixel diameters (7 pixels gave the least amount of error in liposome spot detection). A Boolean mask is generated by setting all pixels in the mean-filtered image that are greater than two times the average value of all the mean-filtered pixels to True and all other pixels to False (this is also an optimized parameter based on the images that were taken). The True areas of the image represent the liposome spots and the False areas represent the background. Spots that lie along the borders of the image are removed, and all False areas completely surrounded by True (i.e. "holes" or "islands") with 8-connectivity are filled in (changed to True). Each separate spot is then labeled with a unique integer. The eccentricity of each spot is examined, and spots that are not circle-like (eccentricity < 0.6) are eliminated from analysis. This is reasonable because DOTAP liposomes are circular, and this will eliminate particles that are touching/overlapped in the image. Finally, the number of pixels in each spot is counted. The distributions are then saved as a comma-separated value (CSV) file.
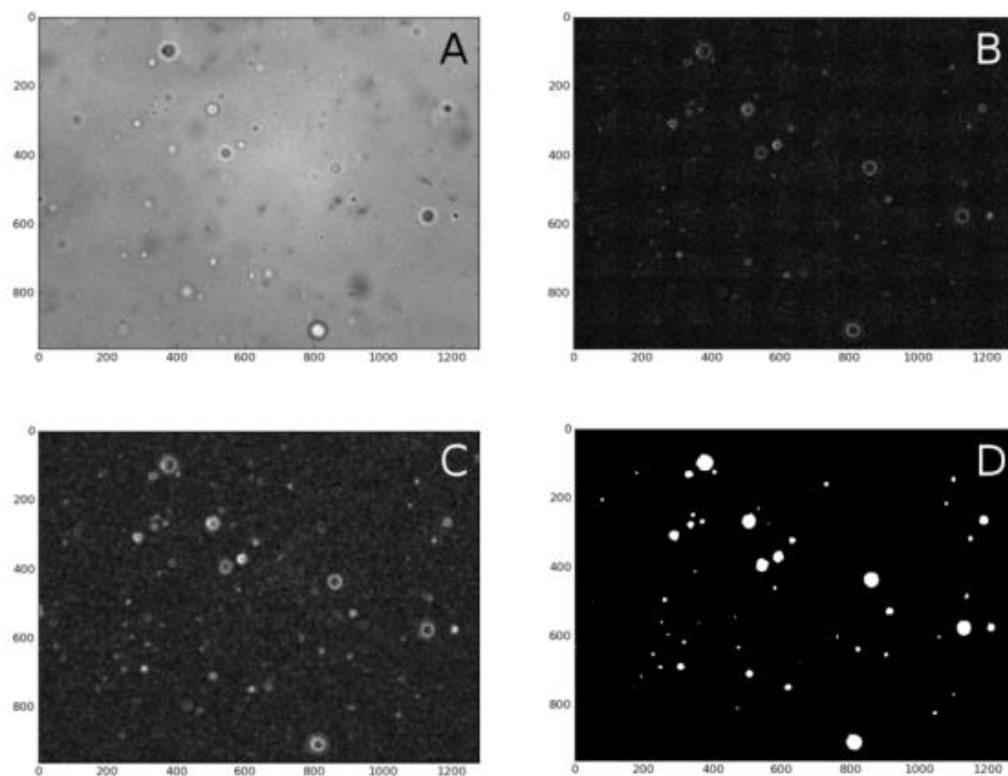
Figure 6. Example Input and Results from Spot Identification Program. A) Original microscopy image of liposomes. B) Sobel-filter of (A). C) Mean-filter of (B). D) Threshold of (C). White areas are "True" and black areas are "False."

This automated method is robust for our particular microscopy image and it was parameterized to keep false positives at a minimum. In-focus spot areas are slightly overestimated. This error arises mainly from the mean filter. Slightly out-of-focus spot areas are slightly underestimated. This is because the peak maxima are not at the very edge of the spots but are offset from the edge closer to the center. This is reasonable since the liposome image grows when it is slightly out-of-focus. Very out-of-focus spots are excluded via the chosen threshold. These biases are consistent throughout the samples that were taken.

Statistical Analysis
Analysis of the particle area distribution CSVs was performed with the JMP statistics software package. The histogram of spot areas showed positive skew. The skew was corrected via the natural log transform before statistical tests were applied.

Two-way ANOVA was performed on spot size data from the accelerated stability study. Both solvent (urine v. water) and timepoints (baseline thru 240 min) were used as model effects. Tukey's Honest Significant Difference (HSD) and t-tests were used for post-hoc analysis.

For the long-term study, one-way ANOVA was performed. Tukey's HSD test was chosen as a post-hoc test to compare spot area data between timepoints.

The α-level for all tests was chosen to be 0.05. Results are reported as the mean log (spot areas) +/- Standard Error of the Mean (SEM).

Results
DOTAP Liposome Size Distributions

The liposomes appear to naturally tend toward a smaller size, but there are some larger liposomes that form upon hydration. Figure 7 (left) shows the distribution of particle sizes via the number of pixels per particle. The distribution shows a strong positive skew. In order to apply statistical analysis appropriately, the data were transformed by taking the log of the spot areas (Fig 7 Right).The log transform provides an approximately normal distribution and provided a better skew correction than the square root, square, cube, and inverse transforms.
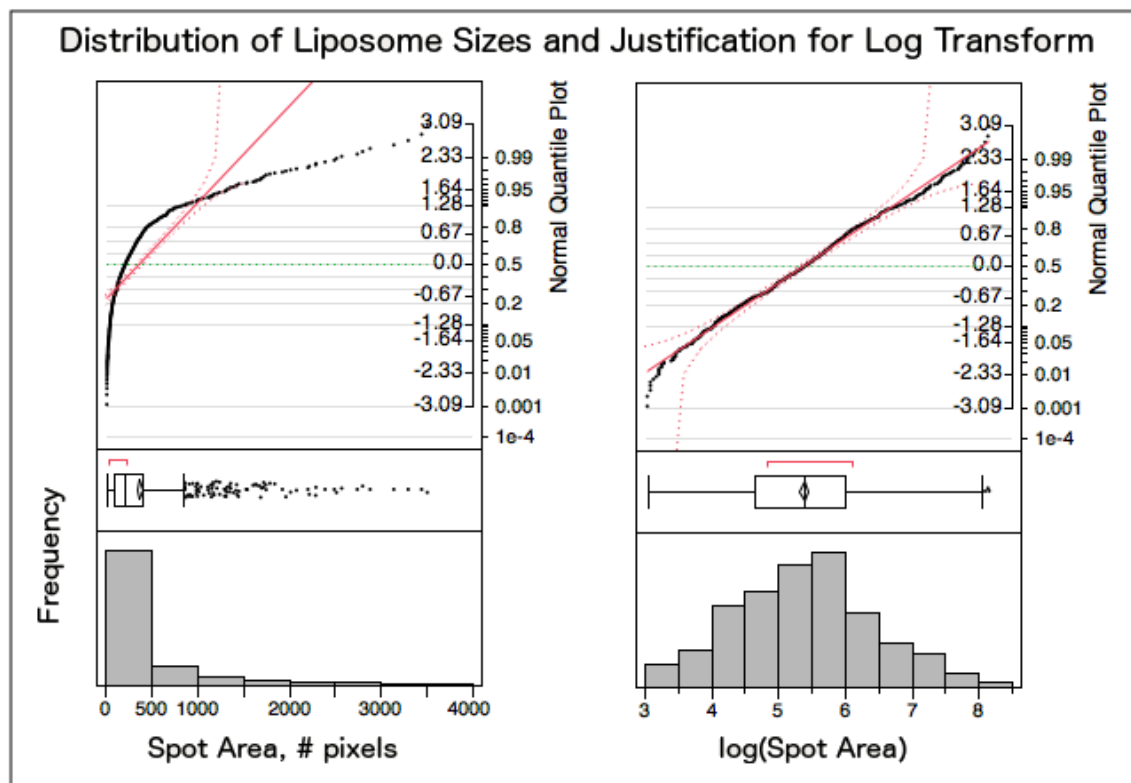
Figure 7. Distribution of Liposome Sizes. Shown here are sample histograms from the baseline data of the long-term stability study (similar distributions were seen in the accelerated study). The left histogram shows the distribution of spot sizes. It shows a strong positive skew and the normal quantile plot suggests that the data are far from a normal distribution. The right histogram shows the result of the log transform applied to the data.
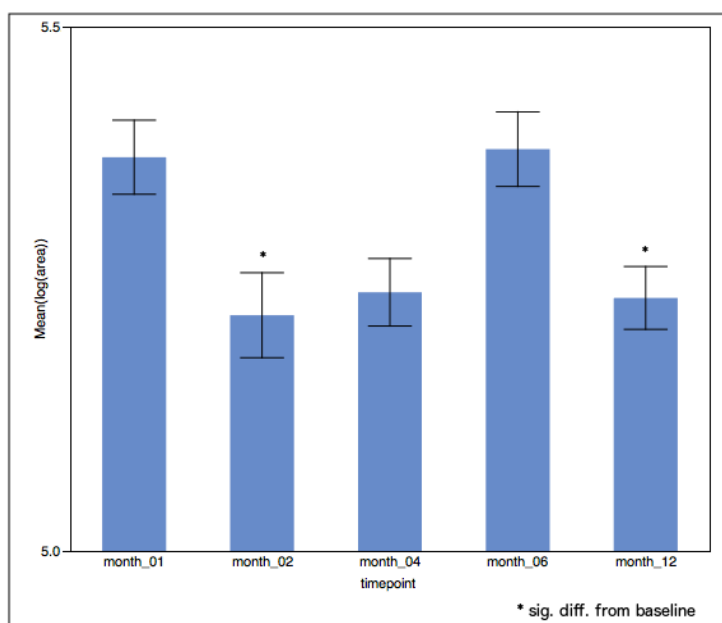
Long-Term Stability Study Results
For the long-term study, the ANOVA test suggested repeated measures of the liposome spot sizes changed significantly (p = 0.0005). Results for mean log (spot areas) and Tukey's HSD test are as follows:

Comparison	p-value, Tukey's HSD
Month 1 vs. Month 2	0.0290*
Month 1 vs. Month 4	0.0579
Month 1 vs. Month 6	0.9999
Month 1 vs. Month 12	0.0295*

* Significant decrease in size from baseline

Timepoint	# LPs	# images	Ave. #LPs/image	Mean log(Pixel Area) +/- SEM
Month 1	851	21	40.5	5.38 +/- 0.03
Month 2	666	23	29.0	5.22 +/- 0.04
Month 4	865	22	39.3	5.25 +/- 0.03
Month 6	807	25	32.3	5.39 +/- 0.04
Month 12	1043	29	36.0	5.24 +/- 0.03

Figure 8. Long Term Stability Study: Mean of log of particle areas. The mean size of the particles decreased significantly from baseline at months 2 and 12.

Discussion

From the results of the accelerated stability study, it appears that the solvent environment can have a significant effect on the size distribution. When placed in synthetic urine, there appears to be a significant decrease in the mean liposome size in the DOTAP/SIRNA formulation. With water as a diluent, there was not a significant change in the mean liposome size over the course of the four-hour stability study. However, there was a significant decrease in the urine sample size distribution from baseline at the 180 min timepoint.

The long-term data suggest that there may be a significant decrease in the mean liposome size over time. A significant decrease from baseline was seen at months 2 and 12, but there was no difference from baseline at months 4 and 6. Different vials were used at each timepoint. It is possible that these effects were due to variability between sample vials. Past experiments (in house; unpublished) with sphingomyelin liposomes showed no variability between liposome vials, but these experiments have not yet been performed for DOTAP liposomes. Further study appears to be necessary to discern activity of the DOTAP liposomes over long timescales.

AIM 2 *Ex-vivo stress testing*
[Accomplishment]
Stress testing was performed to characterize stability of LP-11 liposomes in a bladder environment.

Stress Stability Experiment

Purpose
The purpose of the ex-vivo stress testing was to evaluate the stability of the LP-11 liposomes in a simulated bladder environment since the product is intended for direct intravesical administration and would thus be subject to the chemical environment of the urinary bladder.

Procedure
Two critical product characteristics of the LP-11 liposomes were evaluated following exposure to the simulated bladder environment: particle size and DOTAP concentration. The testing procedure for each of these characteristics was the same as the methods listed above for the long-term stability evaluations. Vials were manufactured to contain 10mg DOTAP liposomes. Each vial was first reconstituted with 1.428mL USP Sterile water. The bladder-like environment was then modeled by mixing the reconstituted liposomes (7mg/mL) in a

2:1 (v/v) with synthetic urine (2.856mL) for a concentration of 2.33mg/mL. Vials were then incubated at 37C for 1 hour to mimic physiologic conditions (clinical protocol will include an instillation time of 1 hour).

Results
The DOTAP component of LP-11 was determined to be consistent under "stress" stability conditions of dilution with synthetic urine and incubation at 37C for 1 hour. Quantitative results are listed in **Table 2** below.

Table 2: Stress Stability Results—Concentration of DOTAP in LP-11 Liposomes in mg/mL by ^{1}H NMR

Timepoint	Concentration (mg/mL)
1 month	2.28

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
See section VII reportable outcomes.

III. Impact
What was the impact on the development of the principal discipline(s) of the project?
The impact of the project included the key accomplishments listed below:
- Validation of the colitis models and development of the new animal model of chronic cystitis
- LP-NGF antisense treatment evaluation in colitis and cystitis rat models
- LP-11 formulation
- Established material controls
- Aseptic manufacturing evaluation
- Pilot production of LP-11
- 12 month product stability and shelf life testing
- Completed product stress testing

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

IV. Changes/Problems

Nothing to report.

V. Product

LP-11

VI. Participants and Collaborating Organizations

Collaborating Organizations
University of Pittsburgh School of Medicine (Pittsburgh, PA) and Ricerca Biosciences, LLC (Concord, OH).

Research Personnel List
Michael Chancellor, Jonathan Kaufman, Michele Gruber, Joe Janicki, Naoki Yoshimura, Pradeep Tyagi, David Chancellor, and Ryan Pruchnic.

VII. Special Reporting Requirements

N/A

VIII. Conclusions

We accomplished the goals of the project which were outlined in the statement of work including: formulating bladder administration of liposomes-NGF antisense (LP-11), confirmation of the efficacy of LP-11 in colitis and cystitis animal models, establishing production capabilities, SOPs and controls for LP-11. Lipella Pharmaceuticals and all of the co-investigators of this project are grateful to the Congressionally Directed Medical Research Programs and the PRMRP Technology/Therapeutic Development Program for the opportunity to advance the development of LP-11 from a concept to a drug candidate nearing initial clinical use. Lipella will continue development of LP-11 in the hopes of addressing the unmet need of IC/BPS.

IX. Reportable outcomes
Publications:
Refereed articles:
1. Kashyap M, Kawamorita N, Tyagi V, Sugino Y, Chancellor M, Yoshimura N, Tyagi P. Down - Regulation of Nerve Growth Factor Expression in the Bladder by Antisense Oligonucleotides as New Treatment for Overactive Bladder. Journal of Urology, 190: 757-764, 2013.
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10. Tyagi, P., Kashyap, M., Hensley, H., Yoshimura, N.: Advances in intravesical therapy for urinary tract disorders. Expert Opinion on Drug Delivery, 13: 71-84, 2016.
11. Kashyap, M., Pore, S., Yoshimura, N., Tyagi, P.: Constitutive expression of NGF and P75NTR affected by bladder distension and NGF antisense treatment. Life Sciences, 148: 93-98. 2016.
12. Kawamorita, N., Yoshikawa, S., Kashyap, M., Tyagi, P., Arai, Y., Chancellor, M.B., Yoshimura, N.: Liposome-based intravesical therapy targeting nerve growth factor (NGF) ameliorates bladder hypersensitivity in rats with experimental colitis. Journal of Urology, 195: 1920-1926, 2016.
13. Dogishi, K., Homan, T., Majima, T., Okamoto, K., Konishi-Shiotsu, S., Kodera, M., Oyama, S., Oyama, T., Shirakawa, H., Yoshimura, N., Nakagawa, T, Kaneko, S.: A rat long-lasting cystitis model induced by intravesical injection of hydrogen peroxide. Physiological Reports, in press, 2017
14. Majima, T., Tyagi, P., Dogishi, K., Kashyap, M., Funahashi, Y., Gotoh, M., Chancellor, M.B., Yoshimura, N: The effect of intravesical liposome-based NGF antisense therapy on bladder overactivity and nociception in a rat model of cystitis induced by hydrogen peroxide. Human Gene Therapy, in press, 2017.

Published abstracts:
1. Kawamorita N, Yoshikawa S, Pradeep T, Chancellor MB, Yoshimura N: Antisense oligonucleotide targeting NGF attenuates bladder pain behavior in rat with experimental colitis. 43rd Annual meeting of the International Continence Society, Abstract No. 148, Barcelona, Spain, August 26-30, 2013. Neurourology and Urodynamics (2013) 32: 727-728.
2. Kawamorita, N., Yoshikawa, S., Arai, Y., Tyagi, P., Chancellor, M.B., Yoshimura, N.: Therapeutic effect of antisense oligonucleotide (OND) targeting nerve growth factor (NGF) on bladder hypersensitivity in rat with experimental colitis. 112th Annual Meeting AUA, PD9-01, Orlando, FL, May 16-22, 2014.
3. Kawamorita N, Yoshikawa S, Tyagi P, Chancellor MB, Yoshimura N: Liposome-based intravesical therapy targeting nerve growth factor (NGF) ameliorates bladder hypersensitivity in rats with experimental colitis. 44th Annual meeting of the International Continence Society, Abstract No. 372, Rio de Janeiro, Brazil, October 20-24, 2014.
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6. Majima, T., Tyagi, P., Dogishi, K., Kashyap, M., Gotoh, M., Chancellor, M., Yoshimura, N.: The impact of intravesical liposome-based NGF antisense therapy on bladder overactivity and nociception in a rat model of cystitis induced by hydrogen peroxide. MP60-03, 114th Annual Meeting AUA, San Diego, CA, May 06-10, 2016.